REMARKS

Claims 61-77 and 83-87 are currently pending in this application, and Claim 49 is withdrawn. In the Office Action mailed November 2, 2005, the claims are rejected under 35 U.S.C. §112(1) and 35 U.S.C. §112(2). Each rejection is addressed below.

I. Rejection of Claims 64-65 and 69 Under 35 U.S.C. §112(2)

Claims 64-65 and 69 are rejected under 35 U.S.C. §112(2) as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. In particular, the Examiner states, "With regard to claims 64-65, is vague and indefinite for the inclusion of other cells expressing cell-surface Hsp70. The claim does not further limit the base claims from which it depends. Claim 69 recites the limitation 'said condition' in line 1. There is insufficient antecedent basis for this limitation in claim 67 from which it depends." Office Action, page 6. The Applicants respectfully disagree.

In a nutshell, Claim 61 describes, for example, a method for the ex vivo activation of NK cells, comprising a suspension of the NK cells together with an Hsp70 protein species. Dependent Claim 64 requires said suspension to further comprise cells expressing cell surface Hsp70 (e.g., a cell species different from NK cells) since NK cells do not express Hsp70. Accordingly, the additional feature of the suspension as characterized in Claim 64 is a clear limitation of Claim 61 on which it depends. Indeed, the additional cells expressing cell surface Hsp70 are different from NK cells. In fact, the cell surface Hsp70-expressing cells are the target cells of the activated NK cells (see, e.g., the description of the present application at page 6, lines 1-8; and Examples 2 and 3; and Fig. 6 which illustrates the embodiment of Claim 64 with respect to Hsp70-expressing tumor cells).

Dependent claim 65 further characterizes said cell surface Hsp70-expressing cells of Claim 64 as, for example, diseased cells from a patient. This additional feature in Claim 65 represents a further limitation of Claim 64, since other cell surface Hsp70-expressing cells, which do not necessarily have to be diseased cells from a patient, are excluded from the claim. In summary, the additional features in Claims 64 and 65 are clearly defined and have a definite meaning. For this reason, it is requested that the rejection of these claims be withdrawn.

Regarding Claim 69, in order to expedite prosecution, the Applicants now amend the claim such that the term "said condition" is replaced with "said contacting." Accordingly, this amendment renders the rejection moot.

II. Rejection of Claims 61-77 and 83-87 Under 35 U.S.C. §112(1)

Claims 61-77 and 83-87 are rejected under 35 U.S.C. §112(1) for failing to comply with the written description requirement. In particular, the Examiner states, "The written description in this case has only set forth a method of activating NK cells using either a HSP protein of SEQ ID NO: 1, a C-terminal fragment comprising amino acids 384-641 of SEQ ID NO: 1, and therefore the written description is not commensurate in scope to the claims that read on a method of activating NK cells using a polypeptide having 70% or greater homology to amino acid 384-641 of SEQ ID NO: 1, as claimed." Office Action, page 3.

A. A Genus of Hsp70 Proteins is Available to One of Skill in the Art

The Applicants respectfully disagree. In accordance with the claimed invention, an Hsp70 species has been used (e.g., rHsp70homC) which is a member of the Hsp70 family exhibiting a homology of about 94% to amino acids 384-561 of Hsp70 protein (SEQ ID NO: 1) and a homology of about 84% to amino acids 384-641 of the reference Hsp70 amino acid sequence (see, e.g., the Specification at page 15, and compare Fig. 4 of document Milner and Campbell, Imnmonogenetics 32 (1990), 242-251 referenced in the specification at page 3, third full paragraph; hereinafter, "the Milner and Campbell reference"). In Fig. 4 of the Milner and Campbell reference the Hsp70homC protein is referenced with "HOM".

For the convenience of the Examiner, a copy of the Milner and Campbell reference is provided herewith to be considered and made of record in the instant application. As demonstrated in Example 1 and shown in Fig. 1A of the present application, the proliferation and thus activity of NK cells could be stimulated by recombinant human Hsp70 as well as by the C-terminal fragment of homologous protein rHsp70homC (see, also, the Specification at page 16, line 2 through the completion of the second full paragraph).

Thus, contrary to the allegation of the Examiner, the Applicants have actually reduced to practice a sequence that is 70% or greater in homology to amino acids 384-641 of SEQ ID NO: 1.

Furthermore, the enclosed Milner and Campbell reference provides evidence that already in 1990, i.e. eight years prior to the effective filing date of the present application, several members of the Hsp70 family were known, which can be used in accordance with the teaching of the present invention.

For example, besides the Hsp70-Hom protein the Milner and Campbell reference discloses in Fig. 4 another homologous Hsp70 protein, i.e. Hsp70-B', the amino acid sequence of which has a homology of about 74% to amino acids 384-641 of the reference Hsp70 protein. Furthermore, reference is made to the publications by the inventor Multhoff et al., Ce11 Stress & Chaperones 1 (1996), 6-1 1 and Multhoff et al., Biol. Chem. 379 (1998), 295-300, both referenced at Page 1 of the present application, which provide an overview concerning Hsp70 multigene family including various citations.

Thus, besides the fact that applicant actually reduced to practice homologous sequences of amino acids 384-641 of SEQ ID NO: 1 the prior art at the time the application was filed provided already a vast source of the genus of Hsp70 sequences that are encompassed in the claimed method.

Even if a person skilled in the art did not want to rely on the available Hsp70 protein species available at the time the present application was filed, the specification provides sufficient guidance how to produce and test appropriate derivatives of the exemplified Hsp70 protein and fragments thereof (see, e.g., the Specification at Page 3, second to fourth full paragraphs, Page 4, the paragraph bridging to Page 5 and Example 1 which describes spanning experiments for testing the capability of Hsp70 species to increase the proliferation of NK cells).

In fact, the pending claims are fully supported by the ample amount of knowledge available in the relevant art when the present application was filed and the guidance provided in the specification.

Furthermore, the claimed invention is not describing a new Hsp70 species, but rather a novel use of Hsp70 family members and C-terminal fragments thereof for the activation of, for example, the immune system and the activation of NK cells. As demonstrated in the specification, the findings of the present invention are especially useful in, for example, tumor

therapy because of the target specificity of the activated immune cells, since the target antigen cell surface Hsp70 is specifically expressed by diseased cells such as tumor cells.

B. The Examiner's Rejection Does Not Comply with the Written Description Guidelines

Applicants respectfully refer the Examiner to the USPTO's "Synopsis of Application of Written Description Guidelines", and in particular to Example 14, pages 53-55. Consideration of the Examples in the Written Description Guidelines establishes that the claims are supported by an adequate written description.

The claim of Example 14 of the Written Description Guidlines recites a protein having SEQ ID NO:3 and variants thereof that are at least 95% identical to SEQ ID NO:3 and catalyze the reaction of A->B. The disclosure of Example 14 provides a single species (SEQ ID NO:3) that has actually been reduced to practice, and describes an assay for identifying the variants having the desired catalytic activity. The analysis considers (1) whether the members of genus vary substantially from each other; and (2) whether the disclosed species is representative of the members of the genus; in order to determine whether one of skill in the art would determine if the applicant was in possession of the necessary common attributes possessed by the members of the genus.

For Example 14, it was determined that the member species did not substantially vary since the variants have 95% identity or greater to the reference sequence, and also possess the catalytic activity. It was also determined that the disclosed species was representative since all members must have at least 95% structural identity to SEQ ID NO:3. The analysis determined that one of skill in the art would conclude that the applicant was in possession of the necessary common attributes possessed by the members of the genus, and therefore the disclosure in this Example meets the written description requirement.

Applicants submit that the instant claims can be analyzed in a similar manner to that provided in Example 14. First, the polypeptides do not substantially vary as members of a genus since they all have at least 70% homology to SEQ ID NO:1 and must possess the activity of inducing an immune response by NK cells. Second, the polypeptide having SEQ ID NO:1 is a representative species of the genus since all polypeptides must have at least 70% homology to this sequence. Therefore, one of skill in the art would conclude that the Applicants were in

possession of the necessary common attributes possessed by the members of the genus, and therefore the instant specification meets the written description requirement for these claims.

CONCLUSION

The Applicants believe that the arguments set forth above traverse the Examiner's rejections and, therefore, request that all grounds for rejection be withdrawn for the reasons set above. Should the Examiner believe that a telephone interview would aid in the prosecution of this application, the Applicants encourage the Examiner to call the undersigned collect at 608-218-6900.

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Structure and expression of the three MHC-linked *HSP70* genes

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Abstract. A duplicated locus encoding the major heat shock-induced protein HSP70 is located in the major histocompatibility complex (MHC) class III region 92 kilobases (kb) telomeric to the *C2* gene. Nucleotide sequence analysis of the two intronless genes, *HSP70-1* and *HSP70-2*, has shown that they encode an identical protein product of 641 amino acids. A third intronless gene, *HSP70-Hom*, has also been identified 4 kb telomeric to the *HSP70-1* gene. This encodes a more basic protein of 641 amino acids which has 90% sequence similarity with HSP70-1. In order to investigate the expression of the three (MHC)-linked *HSP70* genes individually by northern blot analysis, we have isolated locus-specific probes from the 3′ untranslated regions of the genes. The *HSP70-1* and *HSP70-2* genes have been shown to be expressed at high levels as a ~2.4 kb mRNA in cells heat-shocked at 42 °C. *HSP70-1* is also expressed constitutively at very low levels. The *HSP70-Hom* gene, which has no heat shock consensus sequence in its 5′ flanking sequence, is expressed as a ~3 kb mRNA at low levels both constitutively and following heat shock.

Introduction

Heat shock proteins, or stress proteins, are expressed in response to heat shock and a variety of other stress stimuli including oxidative free radicals and toxic metal ions (for reviews see Lindquist 1986; Lindquist and Craig 1988). This response has been observed in all species examined to date. The family of stress proteins of M_r 70000, HSP70, is probably the most predominant and is highly conserved throughout evolution. The amino acid sequence similarities between eukaryotic HSP70s range from 60%-78% and there is 47% similarity between human HSP70 and the *Escherichia coli* homologue *dnaK* (Hunt and Morimoto 1985).

Two independent studies (Goate et al. 1987; Harrison et al. 1987) using somatic cell hybrid analysis and genomic Southern blotting to detect restriction fragment length polymorphisms have demonstrated the presence of multiple autosomal loci for HSP70 in the human genome. These were shown to lie on chromosomes 6, 14, 21, and at least one other autosome. The chromosome 6 *HSP70* loci were localized to a region on the short arm close to the major histocompatibility complex (MHC). During characterization of the class III region of the MHC for additional loci (Sargent et al. 1989a, b) a duplicated locus encoding HSP70 was found between the complement and tumor necrosis factor (*TNF*) genes (Fig. 1A). The *HSP70* loci are 12 kilobases (kb) apart and lie 92 kb telomeric of the *C2* gene. In addition, a region of similarity was localized to a segment of DNA lying ~4 kb telomeric to the first copy of the duplicated *HSP70* locus. *HSP70* loci have also been mapped to the equivalent region of the rat (Wurst et al. 1989) and caprine (Cameron et al. 1990) MHCs.

The human *HSP70* multigene family encodes several highly conserved proteins with structural and functional properties in common, but which vary in their inducibility in response to metabolic stress. The structure of two of these has been completely determined (Wu et al. 1985; Hunt and Morimoto 1985; Leung et al. 1990). The HSP70 sequence determined by Hunt and Morimoto (1985) is encoded by one of the genes (*HSP70-1*) located in the MHC (Harrison et al. 1987; Sargent et al. 1989b). This sequence shares 77% similarity with a more basic HSP70 protein, HSP70B′ (Leung et al. 1990). Other members of the HSP70 protein family include a clathrin uncoating ATPase of M_r 72000 (Chappell et al. 1986) and a glucose-regulated protein (GRP78) of M_r 78000 (Ting and Lee 1988). In addition to the stress-induced proteins, the human genome also contains at least one *HSP70* cognate

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession numbers M34267-9.

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gene (*HSC70*; Dworniczak and Mirault 1987) which is constitutively expressed and shares 82% similarity in its amino acid sequence with *HSP70*.

In order to define the structure of the three MHC-linked *HSP70* loci and to determine whether they were identical to previously identified members of the *HSP70* family, we determined their nucleotide sequences. In addition, we have isolated locus-specific probes from the 3′ untranslated regions of the three genes in order to investigate their expression both constitutively and in response to heat shock.

Materials and methods

Cloning and nucleotide sequence analysis. A 5.6 kb *Eco* RI/*Hind* III fragment containing the *HSP70-1* gene and a 3.6 kb *Pvu* II fragment containing the *HSP70-2* gene were isolated from cos H92 and cos I81 (Sargent et al. 1989a), respectively (Fig. 1B). These fragments were cloned into *Pvu* II-cut pATX and designated pHR-5.6 and pP-3.6, respectively. A 2.4 kb *Bam* HI/*Hind* III fragment containing the coding sequence and 3′ untranslated region of *HSP70-1* and a 0.4 kb *Bam* HI/*Nco* I fragment containing the 5′ flanking and 5′ untranslated region of this gene were isolated from clone pHR-5.4. These were subcloned into *Bam* HI/*Hind* III and *Bam* HI/*Hinc* II-cut Bluescript KS$^+$ vector (clones BS2.4-1 and BS0.4-1). Similarly, a 2.6 kb *Bam* HI/*Pvu* II fragment containing the coding and 3′ untranslated sequence of *HSP70-2* and a 0.9 kb *Bam* HI/*Pvu* II fragment encompassing the 5′ flanking and 5′ untranslated sequence were isolated from pP-3.6 and cloned into *Bam* HI/*Hinc* II-cut Bluescript vector (clones BS2.6-2 and BS0.9-2). The homologous region was isolated from cos H92 in two overlapping fragments: a 4 kb *Bam* HI/*Nco* I fragment which was cloned into *Bam* HI/*Hinc* II-cut Bluescript SK$^+$ (BS4.0-H), and a 1.3 kb *Bam* HI/*Hind* III fragment which was cloned into Bluescript KS$^+$ (BS1.3-H; Fig. 1B). Some smaller fragments from the homologous region were further subcloned into *Sma* I-cut M13 mp10. Single-stranded DNA was recovered from the Bluescript or M13 subclones and sequenced by the dideoxy nucleotide chain termination method using Sequenase (US Biochemicals, Cleveland, Ohio). In the case of the Bluescript clones the helper phage M13K07 was used for ssDNA recovery, in the presence of kanamycin. Nucleotide sequence was obtained using the M13 universal primer or specific oligonucleotide primers, which in the case of the *HSP70-1* and *HSP70-2* genes were based on the sequence published by

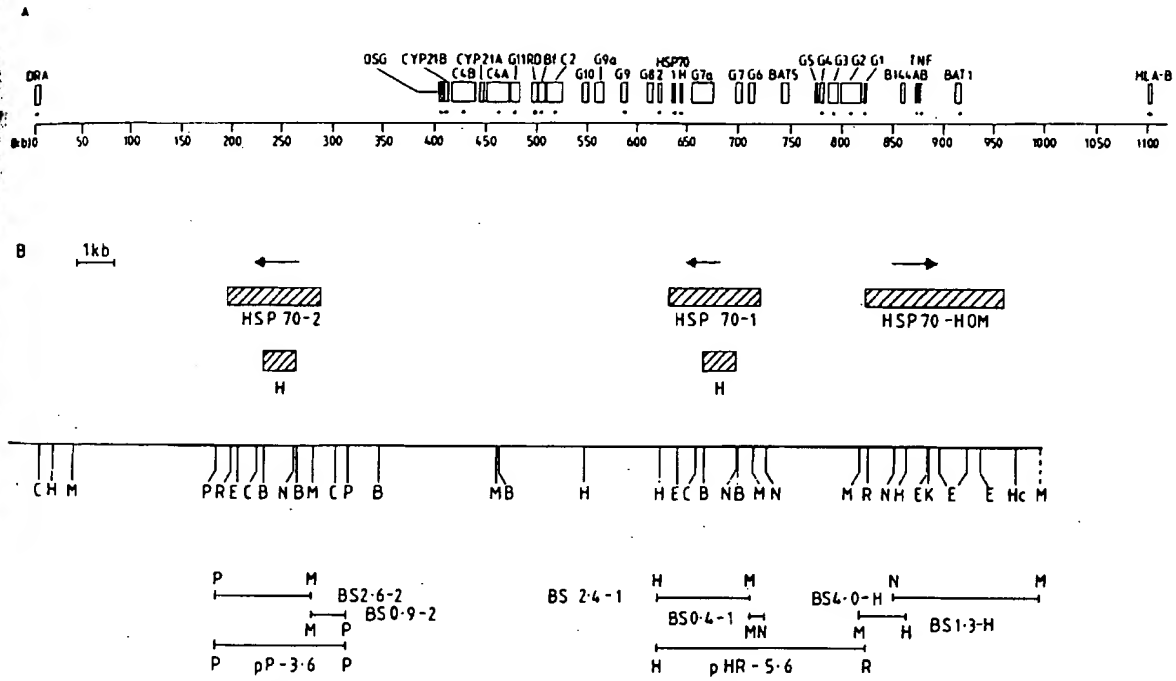

Fig. 1A. Molecular map of the MHC class III region, showing the position of the *HSP70* loci in relation to the other genes identified in this region (Sargent et al. 1989a). *Open boxes* at the *top* of the figures represent the locations of genes. The directions of transcription of the genes are shown by *arrows*, where these have been determined. The *HSP70* loci lie 92 kb telomeric to the *C2* gene. *HSP70-1* and *HSP70-2* are 11 kb apart, and the *HSP70-Hom* gene is 4 kb telomeric to *HSP70-1*. The genes *G2*, *G3*, *G5*, *G7a*, *G9*, *G9a*, and *G10* have also been designated *BAT2*, *BAT3*, *BAT4*, *BAT6*, *BAT7*, *BAT8*, and *BAT9*, respectively (Spies et al. 1989). Details of the *RD* gene and the *OSG* can be found in Levi-Strauss and co-workers (1988) and Morel and co-workers (1989). **B** Restriction map of that part of the MHC class III region containing the *HSP70* loci, which was isolated in overlapping cosmid clones I81 and H92. Restriction sites are shown for the enzymes *Bam* HI (M), *Bgl* II (B), *Cla* I (C), *Eco* 0109 (E), *Eco* RI (R), *Hinc* II (Hc), *Hind* III (H), *Nco* I (N), and *Pvu* II (P). The *Bam* HI site on the *right* of the map, shown by a *dotted line*, was formed by ligation of insert to vector in cos H92. The positions of the *HSP70* loci are shown by *hatched boxes* at the *top* of the figure, and the directions of transcription of the genes are indicated by *arrows*. The 0.9 kb *Bgl* II probe, H, is defined by *hatched boxes*. The *bars* at the *bottom* of the figure represent the regions cloned in pATX and Bluescript vectors.

```
HSP70-1   CGCCATGGAGACCAACACCCTTCCCACCGCCACTCCCCCTTCCTCTCAGGGTCCCTGTCCCCTCCAGTGAATCCCAGAAGACTCTGGAGA   -184
HSP70-2   T       -                       A             -          C                                   -184

HSP70-1   GTTCTGAGCAGGGGGCGGCACTCTGGCCTCTGATTGGTCCAAGGAAGGCTGGGGGGCAGGACGGGAGGCGAAACCCCTGGAATATTCCCG   -94
HSP70-2               A           C   C                                                                -94

HSP70-1   ACCTGGCAGCCTCATCGAGCTCGGTGATTGGCTCAGAAGGGAAAAGGCGGGTCTCCGTGACGACTTATAAAACGCCAGGGGCAAGCGGTC   -4
HSP70-2                           T                     G                AC                 GC G       GC   -4
              ↓
HSP70-1   CGGATAACGGCTAGCCTGAGGAGCTGCTGCGACAGTCCACTACCTTTTTCGAGAGTGACTCCCGTTGTCCCAAGGCTTCCCAGAGCGAAC   87
HSP70-2        A       C                      GG     G  T G C                     CG             T     87

HSP70-1   CTGTGCGGCTGCAGGCACCGGCGCGTCGAGTTTCCGGCGTCCGGAAGGACCGAGCTCTTCTCGCGGATCCAGTGTTCCGTTTCCAGCCCC   177
HSP70-2                             T   T              T C        T         G            C   CCG       177

                                                       M  A  K  A  A  A  I  G  I  D  L  G  T  T  Y  S  C
HSP70-1   CAATCTCAGAGCGGAGCCGACAGAGAGCAGGGAACCGGCATGGCCAAAGCCGCGGCGATCGGCATCGACCTGGGCACCACCTACTCCTGC   267
HSP70-2      G                 C        --     C                                                       265

           V  G  V  F  Q  H  G  K  V  E  I  I  A  N  D  Q  G  N  R  T  T  P  S  Y  V  A  F  T  D  T
HSP70-1   GTGGGGGTGTTCCAACACGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCACCACCCCCAGCTACGTGGCCTTCACGGACACC   357
HSP70-2                                                                                            355

           E  R  L  I  G  D  A  A  K  N  Q  V  A  L  N  P  Q  N  T  V  F  D  A  K  R  L  I  G  R  K
HSP70-1   GAGCGGCTCATCGGGGATGCGGCCAAGAACCAGGTGGCGCTGAACCCGCAGAACACCGTCTTTGACGCGAAGCGCCTGATTGGCCGCAAG   447
HSP70-2                                                                                             C        445

           F  G  D  P  V  V  Q  S  D  M  K  H  W  P  F  Q  V  I  N  D  G  D  K  P  K  V  Q  V  S  Y
HSP70-1   TTCGGCGACCCGGTGGTGCAGTCGGACATGAAGCACTGGCCTTTCCAGGTGATCAACGACGGAGACAAGCCCAAGGTGCAGGTGAGCTAC   537
HSP70-2                                                                                            535

           K  G  E  T  K  A  F  Y  P  E  E  I  S  S  M  V  L  T  K  M  K  E  I  A  E  A  Y  L  G  Y
HSP70-1   AAGGGGGAGACCAAGGCATTCTACCCCGAGGAGATCTCGTCCATGGTGCTGACCAAGATGAAGGAGATCGCCGAGGCGTACCTGGGCTAC   627
HSP70-2                                                                                            625

           P  V  T  N  A  V  I  T  V  P  A  Y  F  N  D  S  Q  R  Q  A  T  K  D  A  G  V  I  A  G  L
HSP70-1   CCGGTGACCAACGCGGTGATCACCGTGCCGGCCTACTTCAACGACTCGCAGCGCCAGGCCACCAAGGATGCGGGTGTGATCGCGGGGCTC   717
HSP70-2                                                                                            715

           N  V  L  R  I  I  N  E  P  T  A  A  A  I  A  Y  G  L  D  R  T  G  K  G  E  R  N  V  L  I
HSP70-1   AACGTGCTGCGGATCATCAACGAGCCCACGGCCGCCGCCATCGCCTACGGCCTGGACAGAACGGGCAAGGGGGAGCGCAACGTGCTCATC   807
HSP70-2                                                                                                C   G    805

           F  D  L  G  G  G  T  F  D  V  S  I  L  T  I  D  D  G  I  F  E  V  K  A  T  A  G  D  T  H
HSP70-1   TTTGACCTGGGCGGGGGCACCTTCGACGTGTCCATCCTGACGATCGACGACGGCATCTTCGAGGTGAAGGCCACGGCCGGGGACACCCAC   897
HSP70-2                                                                                            895

           L  G  G  E  D  F  D  N  R  L  V  N  H  F  V  E  E  F  K  R  K  H  K  K  D  I  S  Q  N  K
HSP70-1   CTGGGTGGGGAGGACTTTGACAACAGGCTGGTGAACCACTTCGTGGAGGAGTTCAAGAGAAAACACAAGAAGGACATCAGCCAGAACAAG   987
HSP70-2                                                                                            985

           R  A  V  R  R  L  R  T  A  C  E  R  A  K  R  T  L  S  S  S  T  Q  A  S  L  E  I  D  S  L
HSP70-1   CGAGCCGTGAGGCGGCTGCGCACCGCCTGCGAGAGGGCCAAGAGGACCCTGTCGTCCAGCACCCAGGCCAGCCTGGAGATCGACTCCCTG   1077
HSP70-2                                                                                            1075

           F  E  G  I  D  F  Y  T  S  I  T  R  A  R  F  E  E  L  C  S  D  L  F  R  S  T  L  E  P  V
HSP70-1   TTTGAGGGCATCGACTTCTACACGTCCATCACCAGGGCGAGGTTCGAGGAGCTGTGCTCCGACCTGTTCCGAAGCACCCTGGAGCCCGTG   1167
HSP70-2                                                                                            1165

           E  K  A  L  R  D  A  K  L  D  K  A  Q  I  H  D  L  V  L  V  G  G  S  T  R  I  P  K  V  Q
HSP70-1   GAGAAGGCTCTGCGCGACGCCAAGCTGGACAAGGCCCAGATTCACGACCTGGTCCTGGTCGGGGGCTCCACCCGCATCCCCAAGGTGCAG   1257
HSP70-2                                                                                            1255

           K  L  L  Q  D  F  F  N  G  R  D  L  N  K  S  I  N  P  D  E  A  V  A  Y  G  A  A  V  Q  A
HSP70-1   AAGCTGCTGCAGGACTTCTTCAACGGGCGCGACCTGAACAAGAGCATCAACCCCGACGAGGCTGTGGCCTACGGGGCGGCGGTGCAGGCG   1347
HSP70-2                A                                                                           1345

           A  I  L  M  G  D  K  S  E  N  V  Q  D  L  L  L  L  D  V  A  P  L  S  L  G  L  E  T  A  G
HSP70-1   GCCATCCTGATGGGGGACAAGTCCGAGAACGTGCAGGACCTGCTGCTGCTGGACGTGGCTCCCCTGTCGCTGGGGCTGGAGACGGCCGGA   1437
HSP70-2                                                                                            1435

           G  V  M  T  A  L  I  K  R  N  S  T  I  P  T  K  Q  T  Q  I  F  T  T  Y  S  D  N  Q  P  G
HSP70-1   GGCGTGATGACTGCCCTGATCAAGCGCAACTCCACCATCCCCACCAAGCAGACGCAGATCTTCACCACCTACTCCGACAACCAACCCGGG   1527
HSP70-2                                                                                            1525

           V  L  I  Q  V  Y  E  G  E  R  A  M  T  K  D  N  N  L  L  G  R  F  E  L  S  G  I  P  P  A
HSP70-1   GTGCTGATCCAGGTGTACGAGGGCGAGAGGGCCATGACGAAAGACAACAATCTGTTGGGGCGCTTCGAGCTGAGCGGCATCCCTCCGGCC   1617
HSP70-2                                                                                            1615

           P  R  G  V  P  Q  I  E  V  T  F  D  I  D  A  N  G  I  L  N  V  T  A  T  D  K  S  T  G  K
HSP70-1   CCCAGGGGCGTGCCCCAGATCGAGGTGACCTTCGACATCGATGCCAACGGCATCCTGAACGTCACGGCCACGGACAAGAGCACCGGCAAG   1707
HSP70-2                                                                                            1705

           A  N  K  I  T  I  T  N  D  K  G  R  L  S  K  E  E  I  E  R  M  V  Q  E  A  E  K  Y  K  A
HSP70-1   GCCAACAAGATCACCATCACCAACGACAAGGGCCGCCTGAGCAAGGAGGAGATCGAGCGCATGGTGCAGGAGGCGGAGAAGTACAAAGCG   1797
HSP70-2                                                                                            1795

           E  D  E  V  Q  R  E  R  V  S  A  K  N  A  L  E  S  Y  A  F  N  M  K  S  A  V  E  D  E  G
HSP70-1   GAGGACGAGGTGCAGCGCGAGAGGGTGTCAGCCAAGAACGCCCTGGACTCCTACGCCTTCAACATGAAGAGCGCCGTGCAGGATGAGGGC   1887
HSP70-2                                                                                            1885
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Fig. 2. Comparison of the nucleotide sequences of the *HSP70-1* and *HSP70-2* genes. The *upper line* shows the sequence of *HSP70-1* and the *lower line* shows nucleotide differences in *HSP70-2*. The transcriptional start site identified by Wu and co-workers (1985) is marked by an *arrow* and defined as position +1. The heat shock consensus elements (GAA/TTC) are indicated by *lines above* the sequence. The distal and proximal CAAT homologies (ATTGG), the TATA box (TATAAAA), and the polyadenylation signals (AATAAA) for both *HSP70-1* and *HSP70-2* are *underlined*. The stop codon (TAG) is indicated by an *asterix*. The derived protein sequence which is identical for both HSP70-1 and HSP70-2 is shown *above* the nucleotide sequence.

Hunt and Morimoto (1985). Oligonucleotides taken from sequence positions 468–489, 762–781, 1084–1101, 1342–1360, 6122–6149, and 1886–1904 (see Fig. 2) were used as sequence primers for the coding regions of both the *HSP70-1* and *HSP70-2* genes. In addition, oligos from sequence positions 2120–2137 in *HSP70-1* and positions 2118–2135 in *HSP70-2* were used to sequence the 3′ untranslated regions.

Southern blot analysis of genomic DNA. Genomic DNA (5 μg) was digested with the appropriate restriction enzyme using the conditions recommended by the supplier (Amersham, Amersham, UK). The digested DNA was fractionated on a 0.8% agarose gel, transferred to nitrocellulose (Southern 1975), and hybridized with ^{32}P-labeled probes. Probes were either labeled directly in LGT agarose, or DNA was purified using GeneClean (Stratech Scientific, London, UK) prior to labeling by random hexanucleotide priming (Feinberg and Vogelstein 1984). Blots were hybridized for 24 h at 42 °C in 50% formamide/5 × Denhardt's solution/10% dextran sulfate/1 *M* NaCl/50 m*M* Tris-HCl pH 7.4/0.1% sodium dodecyl sulfate (SDS) containing 100 μg/ml sonicated salmon sperm DNA. High-stringency washes were performed at 65 °C in 0.1 × standard sodium citrate (SSC)/0.1% SDS for 1 h. Blots were autoradiographed between two intensifying screens at −70 °C for 1–5 days.

Isolation of RNA and northern blot analysis. HeLa and U937 cells were grown in tissue culture to densities of $1–2 \times 10^6$ cells/ml. Cells were either maintained at 37 °C or heat-shocked at 42 °C for 2 h prior to their collection by centrifugation. Some HeLa cells were also fed with 2-deoxy-D-glucose at a final concentration of 10 m*M* for 12 h or starved of serum, by transfer to serum-free media for 24 h, prior to their collection. Total RNA was extracted by guanidinium isothiocyanate lysis and caesium chloride ultracentrifugation (Chirgwin et al. 1979; Maniatis et al. 1982). Polyadenylated poly(A)$^+$ mRNA was isolated from the total RNA by oligodeoxythymidylate chromatography (Maniatis et al. 1982). Samples of total RNA (15 μg) or 2 μg samples of poly(A)$^+$ RNA were fractionated in 0.8% agarose-formaldehyde denaturing gels and transferred onto nitrocellulose (Fourney et al. 1988). Northern blots were hybridized with ^{32}P-labeled probes under the same conditions as genomic Southern blots, as outlined above. High-stringency washing was carried out at 65 °C in 0.2 × SSC/0.1% SDS for 1 h, prior to autoradiography at −70 °C between intensifying screens for 2–10 days.

Results

The HSP70-1 and -2 genes. The complete sequences of the *HSP70-1* and *HSP70-2* genes and their 5′ flanking sequences were determined as described in Materials and methods. The *HSP70-1* gene has an open-reading frame of 1923 base pairs (bp) from the ATG codon at nucleotide 217 to the stop codon (TAG) at nucleotide 2140 (Fig. 2). The predicted relative mass of *HSP70-1* from the derived protein sequence (641 amino acid residues) is 70053. In the 3′ untranslated region the polyadenylation signal AATAAA lies 242 bp from the stop codon. Comparison of the sequence shown in Figure 2 with the previously published *HSP70* gene sequence (Hunt and Morimoto 1985) revealed 11 nucleotide differences. Two of these differences, a C−G transversion at nucleotide 190 and a G insertion at nucleotide 215, lie in the 5′ untranslated region. The remaining nine differences lie within the coding sequence. Of these, two single-base changes predict changes in the derived amino acid sequence at position 7 (GTC−ATC; Val−Ile) and at position 371 (GGC−GCC; Gly−Ala), in agreement with the results of Sargent and co-workers (1989b). The presence of three single-base insertions at nucleotides 1620, 1622, and 1623 in our sequence predicts an extra codon at position 1621. The sequence of nucleotides 1618–1626 in Figure 2 is 5′CCCAGGGGC3′ and encodes Pro-Arg-Gly. The sequence of Hunt and Morimoto (1985) is 5′CCAGGC3′ and encodes Pro-Gly. The additional Arg in our sequence is also found in Drosophila HSP70, *E. coli dnaK*, and HSP70B′ (Fig. 4). The differences between our sequence and that determined by Hunt and Morimoto (1985) could represent allelic polymorphism in the *HSP70-1* gene.

The *HSP70-2* sequence contains a continuous open-reading frame extending from nucleotides 215 to 2138 (Fig. 2), and like the *HSP70-1* gene lacks introns. The derived protein sequence of 641 amino acid residues is identical to that of HSP70-1. The eight nucleotide differences at positions 436, 799, 802, 1267, 1923, 2074, 2119, and 2134 (nucleotide positions refer to HSP70-2) lie in the second or third base of a codon and do not cause alteration in the derived amino acid sequence (see Fig. 2). The G→A transition at position 1267 in HSP70-2 results in the loss of a *Pst* I restriction site. This is the basis for the *Pst* I polymorphism defined by Goate and co-workers (1987). In the 3′ untranslated region of HSP70-2 the polyadenylation signal lies 361 bp from the stop codon. Comparison of the 3′ untranslated region of the *HSP70-1* gene with the equivalent region of the *HSP70-2* gene reveals that they are completely divergent (Fig. 2).

The 5′ untranslated region of the *HSP70-1* gene is 217 bp and is identical to that determined by Hunt and Morimoto (1985) except for the two single-base changes mentioned above. Although the transcriptional start site of the *HSP70-2* gene has not been determined, it is likely that it is at a similar position to that of the *HSP70-1* gene. In primer extension experiments reported by others (Wu et al. 1985), only a single extended product was defined. Given that both genes are transcribed into abundant mRNA species after heat shock (Fig. 6) and that the two sequences differ by only 26 single-base changes, any primer extension based on *HSP70-1* mRNA with the primers used would also have occurred on *HSP70-2* mRNA. In addition, this would place the TATA box sequence at the appropriate place in the *HSP70-2* gene from the transcriptional start site.

Comparison of the 5′ flanking sequence of the *HSP70-1* and -2 genes reveals an additional 17 single-base changes extending back as far as nucleotide -273. However, none of the differences seriously disrupt any of the elements so far identified in the *HSP70-1* gene as being important for the basal level of transcription of the gene, or for transcriptional activation after stress (Wu et al. 1986; Morgan et al. 1987; Amin et al. 1988; Williams et al. 1989; Taylor and Kingston 1990).

The homologous region. The 0.9 kb *Bgl* II probe (H), derived from the *HSP70-1* gene, in addition to hybridizing to the *HSP70-1* and *HSP70-2* genes on Southern blot analysis, was found to hybridize less strongly to a region lying ~4 kb telomeric to the *HSP70-1* gene (Sargent et al. 1989b). In order to define the structure of the homologous region the sequence of 3330 bp of DNA was determined (Fig. 3). This indicated that the homologous region represents another gene (*HSP70-Hom*) of the *HSP70* family. The *HSP70-Hom* gene contains an open-reading frame of 1923 bp from the ATG codon at nucleotide 960 to the stop codon (TAA) at nucleotide 2883. Like the *HSP70-1* and -2 genes, it lacks introns. The 3′ untranslated sequence contains a polyadenylation signal ATTAAA 405 bp from the stop codon and differs significantly from that of the *HSP70-1* and -2 genes. Upstream of the ATG codon at nucleotide 960 are two Alu repeat sequences between nucleotides 294 and 900. A TATA box is found at nucleotide 162. However, no CAAT box sequence or heat shock consensus element with GAA/TTC repeats is found up to 959 bp from the ATG codon.

Comparison of the amino acid sequences of HSP70-Hom and HSP70-1 (Fig. 4) reveals that they are 90% similar. If conservative replacements are taken into account the sequence similarity increases to 94%. The greatest sequence divergence between these two proteins is at the C terminus. The similarity in the C-terminal 100 amino acids is only 72%. There is also a high level of protein sequence identity between HSP70-Hom and HSP70-B′ (79%; Leung et al. 1990) and between HSP70-Hom and HSC70 (84%; Dworniczak and Mirault 1987). Again the sequences differ most in the C-terminal 100 amino acids, where the percentage similarity decreases to 47% between HSP70-Hom and HSP70B′, and to 64% between HSP70-Hom and HSC70.

Southern blot analysis. The human genome contains multiple copies of expressed genes and unexpressed pseudogenes in the *HSP70* family. Due to the high degree of sequence similarity between the coding regions of the *HSP70* genes, DNA probes corresponding to coding regions tend to cross-hybridize to each other. This is apparent in Figure 5A where probe H, in addition to hybridizing to the 2.4 kb, 5 kb, and 7 kb *Bam* HI/*Hind* III fragments which correspond to the *HSP70-1*, *HSP70-Hom*, and *HSP70-2* genes, respectively, also hybridized to other fragments corresponding to *HSP70* genes elsewhere in the genome. In order to differentiate between the various genes in genomic Southern blot analysis and to provide evidence for the expression of the MHC-linked *HSP70* genes in northern blot analysis, it was necessary to define probes corresponding to the regions of minimum sequence similarity between the various genes. Given that the 3′ untranslated regions of each of the three genes showed little sequence similarity to each other or to the 3′ untranslated regions of the *HSP70B′* (Leung et al. 1990) and *HSC70* (Dworniczak and Mirault 1987) genes, the isolation of suitable locus-specific probes was possible.

A 510 bp *Eco* 0109/*Hind* III fragment, a 600 bp *Eco* 0109 fragment, and a 225 bp *Eco* 0109 fragment were isolated from the 3′ untranslated regions of the *HSP70-1*, *HSP70-2*, and *HSP70-Hom* genes, respectively. Each of these probes was hybridized to a Southern blot of genomic

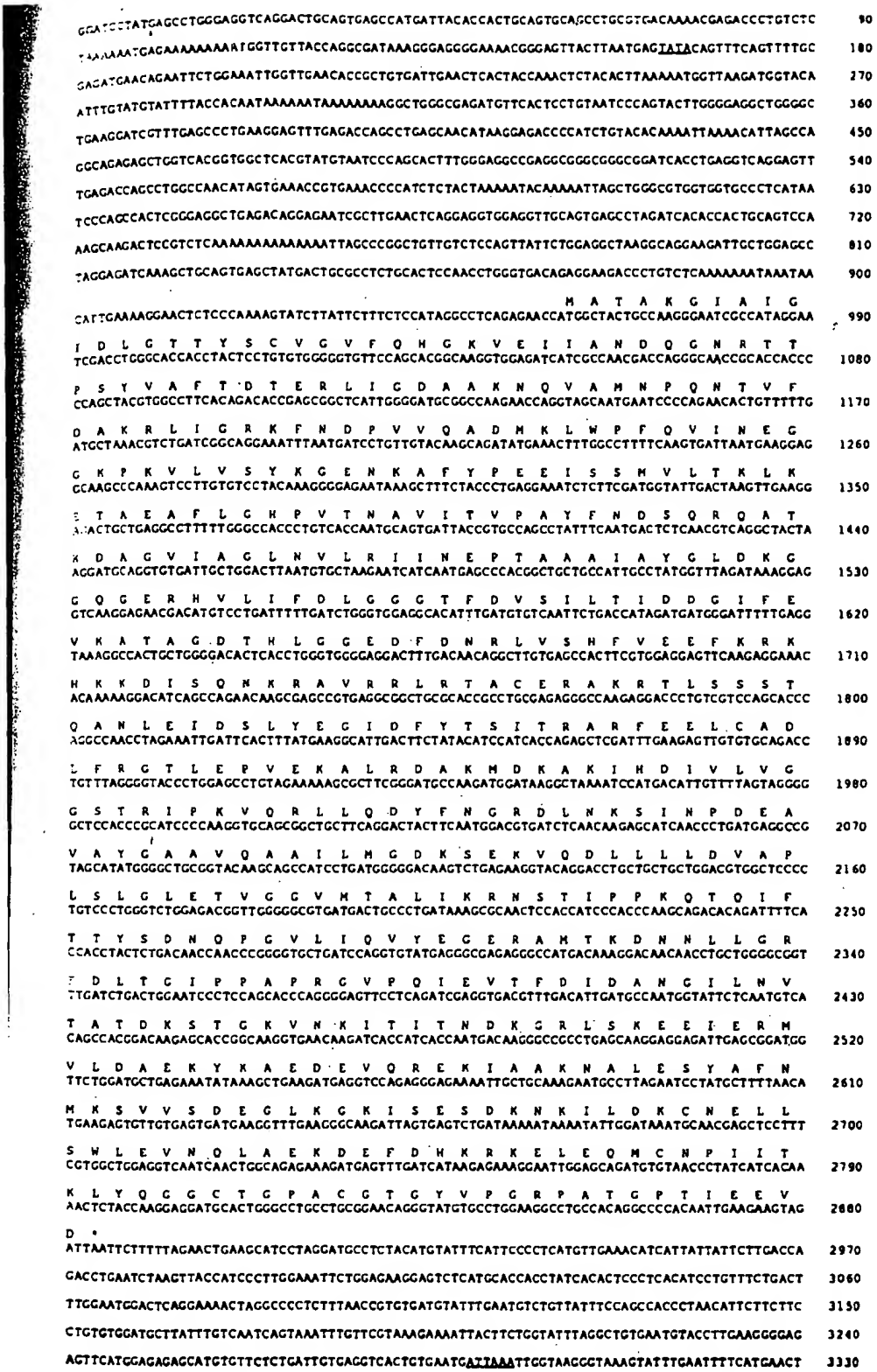

Fig. 3. Sequence of the *HSP70-Hom* gene. The TATA box and polyadenylation signal (ATTAAA) are *underlined*. The stop codon (TAA) is indicated by an *asterix*. The derived protein sequence is shown *above* the nucleotide sequence.

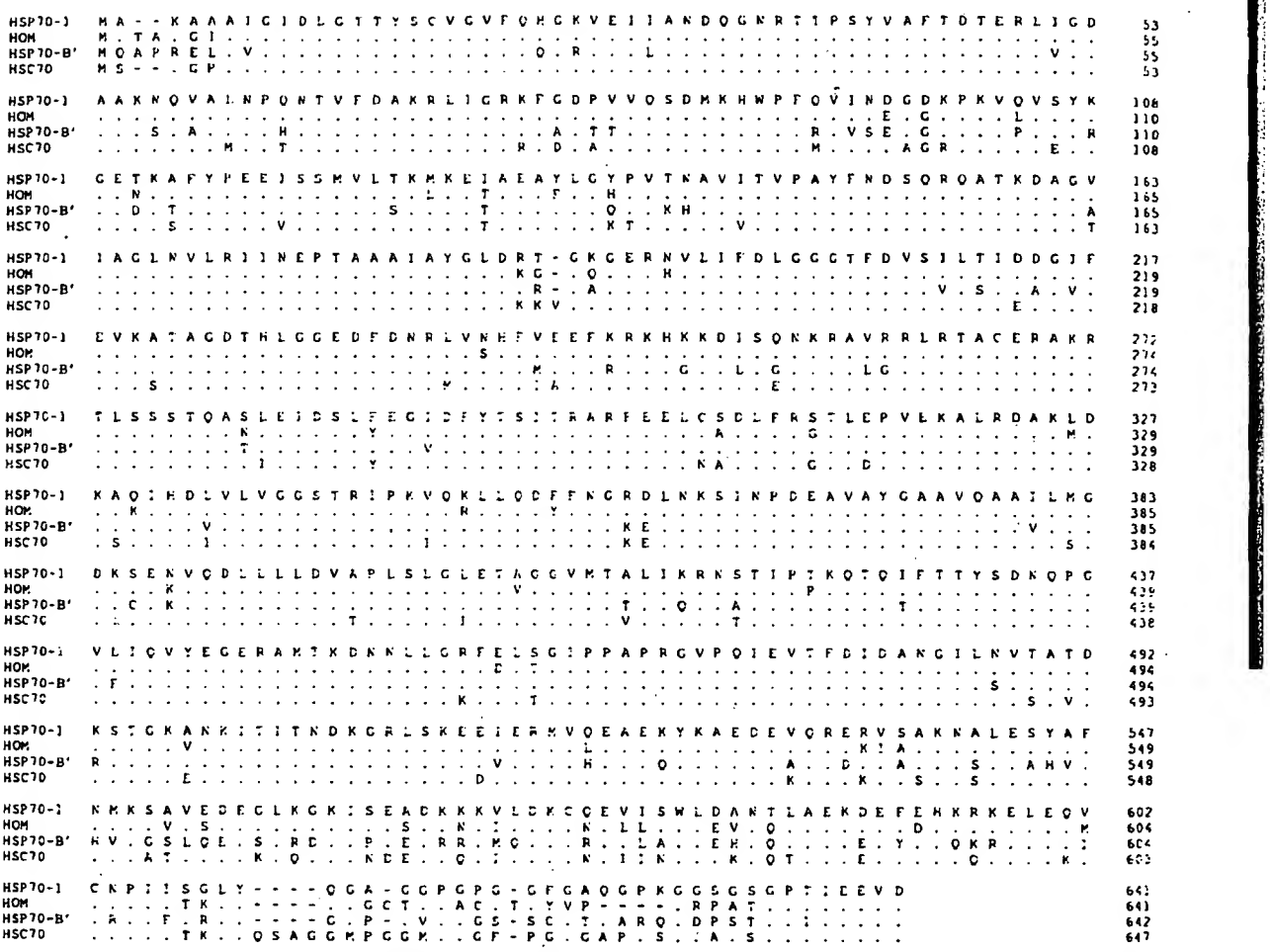

Fig. 4. Comparison of the protein sequences of HSP70-1 and HSP70-Hom with those of the stress-induced protein HSP70-B' (Leung et al. 1990) and the constitutive protein HSC70 (Dworniczak and Mirault 1987). Amino acid residues that differ from HSP70-1 are shown and identical amino acids are indicated by a *dot*. *Dashes* are inserted to optimize alignment of the protein sequences.

DNA digested with *Eco* RI, *Bgl* II, *Bam* HI, and *Hind* III in single- and double-digest combinations. In all cases the probes hybridized uniquely to fragments associated with the *HSP70* locus from which they had been isolated, and to no other fragments (Fig. 5B).

Northern blot analysis. The locus-specific probes described above were used to examine the expression of the MHC-linked *HSP70* genes. Northern blots were prepared using total RNA from HeLa cells either maintained at 37 °C, heat-shocked for 2 h at 42 °C, fed with 2-deoxy-D-glucose for 12 h, or serum-starved for 24 h prior to harvesting. In addition, poly(A)$^+$ RNA from control and heat-shocked U937 cells was used.

With both the *HSP70-1* and *HSP70-2* locus-specific probes an elevated level of a ~2.4 kb mRNA was observed from the heat-shocked HeLa (Fig. 6) and U937 cells (data not shown). A very low level of *HSP70-1* mRNA was detectable in the control cells, but this was not observed for *HSP70-2* (Fig. 6). However, neither 2-deoxy-D-glucose treatment nor serum starvation had any apparent effect on the level of message observed constitutively, in agreement with the results of others (Wu and Morimoto 1985; Wu et al. 1985; Watowich and Morimoto 1988). Assuming that the *HSP70-1* and *HSP70-2* genes share the same transcriptional start site (defined by Wu et al. 1985), the positions of the polyadenylation signals in these two genes predict an mRNA for *HSP70-2* ~100 bp longer than that for *HSP70-1*. The resolution available by northern blotting is not sufficient for this difference to be seen.

When the *HSP70-Hom* locus-specific probe was used to probe northern blots a very low level of an mRNA of ~3 kb was detected from control HeLa cells (Fig. 6) and also heat-shocked, 2-deoxy-D-glucose-treated, and serum-starved cells, a result which indicates that this gene is

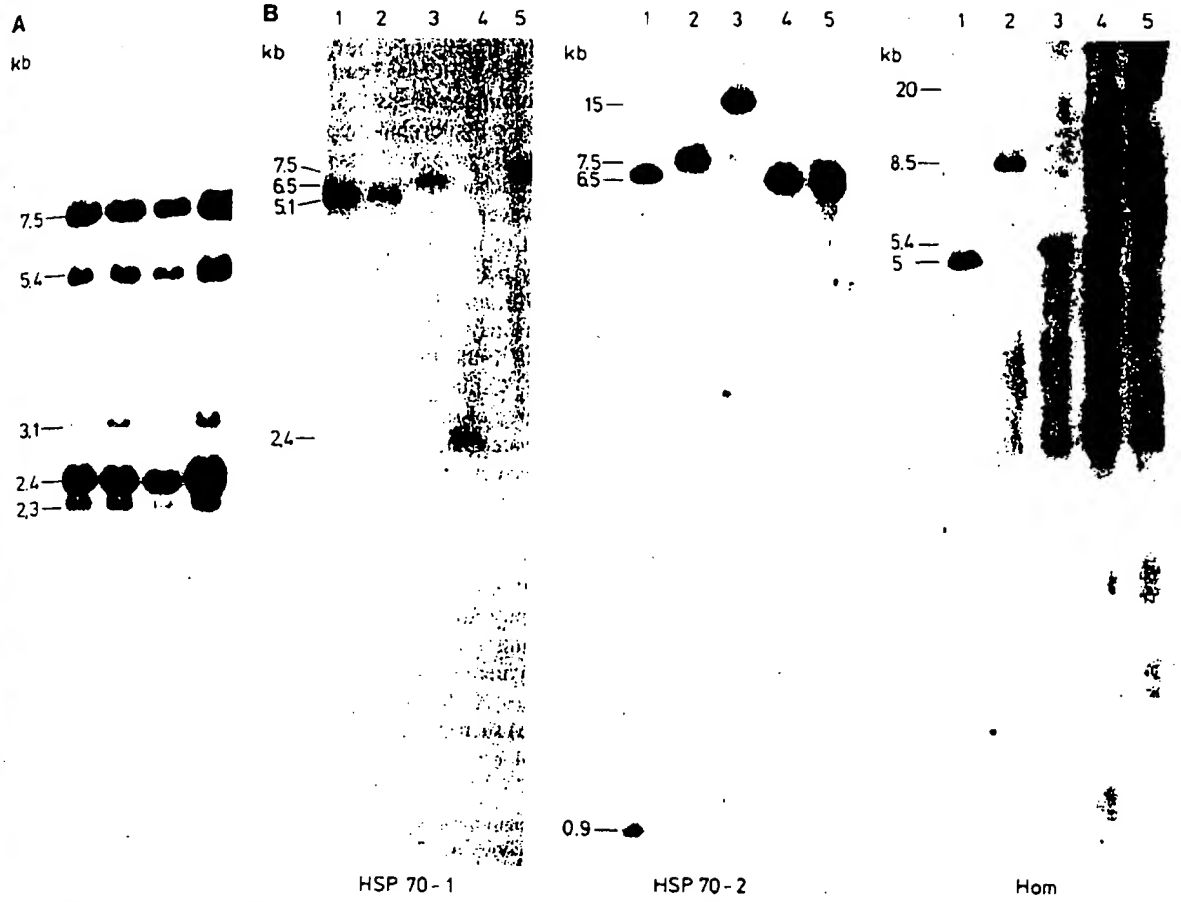

Fig. 5. A, B. Southern blot analysis. A A Southern blot of *Bam* HI/*Hind* III-digested genomic DNA (5 μg) from four individuals was hybridized with the ^{32}P-labeled 0.9 kb *Bgl* II probe (H). After high-stringency washing and autoradiography the probe was found to hybridize strongly to fragments of 7.5, 5.4, and 2.4 kb, which correspond to the MHC-linked *HSP70* loci. In addition, probe H hybridized less strongly to fragments of 3.1 and 2.3 kb, associated with *HSP70* loci elsewhere in the genome. B Locus-specific probes for the MHC-linked *HSP70* loci. Southern blots of genomic DNA (5 μg) from an *HLA*-homozygous cell line (ICE 5) digested with *Eco* RI/*Bgl* II (1), *Bgl* II (2), *Hind* III (3), *Bam* HI/*Hind* III (4), and *Bam* HI (5) were hybridized with the ^{32}P-labeled locus-specific probes prepared from the 3′ untranslated regions of the MHC-linked *HSP70* genes. After high-stringency washing and autoradiography, each of the probes hybridized only to fragments shown by cosmid mapping to be associated with the genes from which they had been derived. Sizes of fragments are shown on the *left* of each blot.

expressed constitutively at a low level but is not induced by heat shock, 2-deoxy-D-glucose, or serum starvation. Although the transcriptional start site of the homologous gene has not been determined, an mRNA of ~3 kb is consistent with the positions of the TATA box (nucleotide 162) and polyadenylation signal (nucleotide 3290).

Discussion

Our analysis of the MHC-linked *HSP70* loci has shown the presence of two intronless genes, *HSP70-1* and *HSP70-2*, which encode an identical protein product and which both express elevated levels of a ~2.4 kb mRNA following heat shock. The observation that two closely linked *HSP70* loci both express an identical protein is somewhat surprising. One possible explanation is that the range of stress factors which induce HSP70 expression is too great for the promotor sequence of a single gene to recognize them all. Therefore, two genes with different 5′ promotor sequences, as in the case of *HSP70-1* and *HSP70-2*, are required to provide an adequate response. In addition, there may be differences in the expression of the two genes in different cell types due to the sequence differences in the promoter regions. Also, the sequences in the 3′ untranslated region which are completely divergent between *HSP70-1* and *HSP70-2* may confer different regulation on the two mRNA species.

Lying ~4 kb telomeric of *HSP70-1* is a third intronless gene, *HSP70-Hom*, which encodes a protein with 90% similarity to HSP70-1. The *HSP70-Hom* gene appears to be constitutively expressed, but levels of its mRNA are not increased following heat shock. This novel

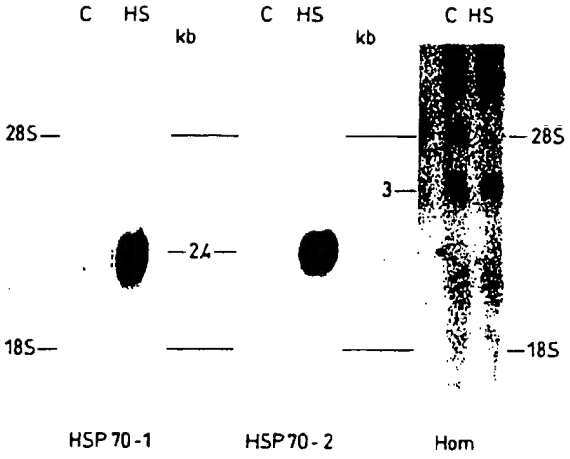

Fig. 6. Northern blot analysis. Total cytoplasmic RNA (15 μg) derived from HeLa cells, either maintained at 37 °C or heat-shocked for 2 h at 42 °C prior to harvesting, was fractionated on 1% agarose-formaldehyde denaturing gels and transferred onto nitrocellulose to give three identical blots. The northern blots were hybridized with the three *HSP70* locus-specific probes. The position of migration of the 28S and 18S RNA is shown. The *HSP70-1* probe detected a transcript of ~2.4 kb at a very low level in constitutive RNA (C) and at a much elevated level in heat-shocked RNA (HS). The *HSP70-2* probe detected no transcript in constitutive RNA (C), and a high level of a ~2.4 kb transcript in heat-shocked RNA (HS). The homologous gene probe detected low levels of a ~3 kb transcript in constitutive (C) and heat-shocked (HS) RNA.

HSP70 gene encodes an HSP70 variant that is more basic than HSP70-1, and is distinct from HSC70 and the recently described HSP70B′ (Leung et al. 1990), which are encoded by genes in other parts of the genome.

Comparison of HSP70-1 and HSP70-Hom with other members of the HSP70 protein family reveals that they are highly similar (79%–84%). The greatest divergence is in the C-terminal 100 amino acids (HSP70-Hom vs HSP70-1, 72%; HSP70-Hom vs HSP70B′, 47%; HSP70-Hom vs HSC70, 64%) and this may indicate a selective function of the different HSP70 proteins.

Transcription of the *HSP70-1* gene has been shown to be induced by serum stimulation after serum starvation (Wu and Morimoto 1985). However, serum withdrawal alone has been shown to result in increased levels of a cytosolic protein, prp 73, in rats (Chiang et al. 1989). This protein has been shown to be a member of the HSP70 family and has been found to bind to peptide regions that target intracellular proteins for lysozomal degradation, in response to serum withdrawal. We therefore looked for elevated levels of mRNA from the MHC-linked *HSP70* genes following serum withdrawal, but this was not observed. Elevated levels of mRNA from the glucose-responsive *HSP70* family member *GRP78* (*BiP*) have been shown to occur following treatment with 2-deoxy-D-glucose (Watowich and Morimoto 1988). However, elevated levels of mRNA were not detected for any of the MHC-linked *HSP70*, following 2-deoxy-D-glucose treatment of cells in culture.

The MHC has been shown to influence more than 40 autoimmune diseases. In some cases, a direct involvement of the polymorphic class I and class II and the less variable class III gene products has been proposed (Batchelor and McMichael 1987; Todd et al. 1988). In many cases, however, the role of the MHC is unclear. There is a great deal of interest in the possible roles that stress proteins may play in immune responses (for review see Young 1990), and there is evidence to suggest that HSP70 proteins may be involved in autoimmune diseases (van Eden et al. 1988; Res et al. 1988; Lamb et al. 1989; Yasuhiro and Kishimoro 1990). However, due to the very high degree of sequence similarity it is not easy to distinguish between the different HSP70 family members using cDNA or genomic probes. Thus, the locus-specific probes reported here will make it possible to investigate variations in gene copy number and variations in the levels of expression of the MHC-linked *HSP70* genes in relation to those *HLA* types frequently associated with autoimmunity. In addition, the sequence differences in the 5′ and 3′ untranslated regions allow the specific amplification by polymerase chain reaction of the MHC-linked *HSP70* genes (C. M. Milner and R. D. Campbell, unpublished data) such that sequence variation in these genes between haplotypes can be investigated.

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